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(54) Title: CULTURING BONE MARROW CELLS FOR ADOPTIVE IMMUNOTHERAPY

(57) Abstract

In the field of bone marrow transplantation and adoptive immunotherapy, methods for *in vitro* culturing therapeutic quantities of bone marrow cells are provided. Bone marrow cells, which may be autologous or obtained from a donor, are cultured in a hollow fiber bioreactor culture system. The bone marrow cells may be pre-treated with a chemotherapeutic or other agent, or cultured in the presence of at least one chemotherapeutic or other agent or growth promoting substance. Heterologous DNA may be introduced into the bone marrow cells during culture. Bone marrow cells cultured according to the methods of the invention may be used in adoptive immunotherapy procedures to treat cancer, genetic disorders and AIDS.

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CULTURING BONE MARROW CELLS FOR ADOPTIVE IMMUNOTHERAPY

This invention is a continuation-in-part of U.S. application serial number 07/407,456, which was filed on 5 September 14, 1989 and which is a continuation-in-part of U.S. application serial number 07/238,445, which was filed on August 31, 1988. The subject matter of these applications is incorporated herein by reference thereto.

Unless defined otherwise, all technical and 10 scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference thereto.

FIELD OF INVENTION

15 This invention is directed to methods for culturing bone marrow cells in vitro in a hollow fiber bioreactor, to the cultured bone marrow cells and to methods of adoptive immunotherapy using the cultured bone marrow cells. The bone marrow cells that are cultured in 20 the bioreactor are obtained from normal individuals and from individuals suffering from various diseases including leukemias, metastatic cancers, and genetic disorders.

BACKGROUND OF THE INVENTION

25 A recent and highly promising development in the therapeutic treatment of diseases involves the use of adoptive immunotherapy (See, e.g. Belldegrun et al. (1989) Chapter 12, in Urologic Oncology, Lepor et al. (eds.), Kluwer Academic Publishers, Boston). Adoptive 30 immunotherapy is the passive transfer to an individual, who is suffering from an acquired or inherited disease, of immunologically active cells, which have been removed

from the individual or from a donor. Often the immunologically active cells are manipulated and/or modified in vitro prior to transfer to the recipient.

The transferred cells are used therapeutically 5 to treat the disease via destruction of the affected cells of the recipient by virtue of specific interaction with the affected cells of the recipient, by replacement or supplementation of the affected cells or by furnishing therapeutically effective substances or immunologically 10 active cells to the recipient.

Methods of adoptive immunotherapy involving the transfer of lymphoid cells that have been cultured in vitro in a bioreactor under conditions in which specific 15 subpopulations of lymphoid cells are selectively expanded are described in U.S. Patent Application No. 07/407,456 to Knazek et al., which has been herein incorporated in its entirety by reference thereto. Lymphoid cells are, however, only a subset of immunologically active cells that may be used in methods of adoptive immunotherapy. 20 Bone marrow cells are also used in methods of adoptive immunotherapy. Bone marrow contains an array of immunologically active cells, including pluripotent stem cells from which all cells of the hematopoietic and immune systems can be reconstituted.

25 The potential uses of adoptive immunotherapy are almost limitless. Not only can it be used for the treatment of cancer, but it can be used for genetic therapy, and as a means of delivering antitumor agents and other therapeutic agents. Presently, lymphoid cells 30 are being explored as a means for introducing genetically engineered DNA into an individual for genetic therapy or to deliver therapeutic agents (see, e.g., Genetic Eng.

News. Vol. 9, No. 3, March 1989 and p. 133 in Business Week/May 1, 1989). It has been proposed that lymphocytes will be removed from the patient and DNA that encodes a wild-type protein for which the individual is 5 deficient or that encodes a therapeutically effective anticancer or antiviral agent, such as interferon or tumor necrosis factor (TNF), will be introduced into lymphocytes in a manner such that the encoded protein will be expressed. The lymphocytes will then be 10 reintroduced into the patient and the heterologous DNA will be expressed.

Lymphocytes, however, are not ideal candidates for genetic therapy because they are differentiated end-stage cell populations, which have limited life 15 expectancies, and, thus, cannot be used to permanently introduce heterologous DNA into an individual. An undifferentiated stem cell, which is self-renewing, such as a pluripotent stem cell from the bone marrow, is a more suitable candidate for genetic therapy. Because of 20 difficulties in culturing such cells, it has not, however, as yet been possible to introduce heterologous DNA into such cells.

Bone marrow, hematopoiesis and lymphopoiesis.

Bone marrow of adult animals, which is found 25 within all of the hollow bones of the body, serves as source of transplantable pluripotent stem cells. Pluripotent stem cells (often abbreviated CFU-S), which also exist in the liver and spleen of adult mammals, have the ability to both proliferate and to differentiate into 30 multipotent cells (see, e.g., Schrader et al. (1978) J. Exp. Med. 148: 823). Multipotent cells potentially, which can develop into fewer lineages than the

pluripotent stem cells and, are, thus of restricted potency, have the ability to both proliferate and differentiate into cells of more restricted potency (see, e.g. Fig. 1, Dexter (1987) British Med. J. 295: 1192-1194). For example, erythroid, myeloid, and lymphoid cells are derived from stem cells that give rise to erythrocytes, granulocytes/macrophages, and lymphocytes, respectively. These latter cells represent the end-stage of differentiation and appear to lack self-renewal potential. The process by which the undifferentiated pluripotent stem cells of the bone marrow develop into the various component cell types of the blood is called hematopoiesis.

Adult bone marrow is the major site of hematopoiesis. The pluripotent stem cells proliferate and differentiate on a framework, called the stromal network, that contains fat cells, fibroblasts, macrophages, blood vessels and sinusoids that coalesce into the marrow venous drainage system. A single pluripotent stem cell can give rise to cells of any lineage.

The more differentiated progenitor cells present in bone marrow can be identified by their respective physical properties, such as distinctive colony phenotype, sedimentation velocity, and density. For example, one type of progenitor cell, which gives rise to erythroid cells is called a "burst forming unit" (BFU-E) because of the distinctive multicentric colonies of erythroid cells that form when BFU-Es are cultured on agar. The cells can also be identified by the distinctive array of cell surface antigens carried by each cell type so that monoclonal antibodies that

specifically recognize a particular cell surface antigen can be used to identify a particular cell type or a particular differentiated state. The presence and numbers of pluripotent stem cells in a bone marrow sample 5 can be assessed by colony formation assays. The length of time it takes for colonies to form and the type of cell formed are indicative of both the presence and relative numbers of pluripotent cells present in a sample.

Lymphopoiesis refers to the process by which 10 CFU-S differentiate into lymphocytes. Lymphocytes first appear in the yolk sac and liver of a developing embryo. After birth they are found in the bone marrow where they persist for life. T and B cells are the principal classes of lymphocytes and the thymus and bone marrow are 15 the primary lymphatic organs. The spleen and lymph nodes are the secondary lymphatic organs. Both T and B lymphocytes, which are ubiquitous in the blood, lymph and connective tissues, are regenerated in the bone marrow from pluripotent stem cells. Prothymocytes are produced 20 in the bone marrow and migrate to the thymus where, under the influence of thymus-produced hormones and growth factors, they proliferate and differentiate into T-cell subpopulations. Among these subpopulations, which are phenotypically distinguished by characteristic cell 25 surface antigens, are helper T cells (T_h), suppressor T cells (T_s) and cytotoxic killer cells (CTLs). For example, mouse T helper (T_h) cells are characteristically $Thy1^+Lyt1^+2^+3^-$, T suppressor (T_s) cells are $Thy1^+Lyt1^-2^+3^+$, and cytotoxic lymphocytes (CTL) are $Thy1^+Lyt1^+2^+3^+$. 30 After trafficking through the thymus, T-cell precursors develop into immunologically active effector and regulatory T-cells. Mature T-cells release factors

that regulate growth and differentiation of both B and T cells in the bone marrow.

B and T lymphocytes, which differ in some surface antigens, appear morphologically similar. They 5 are small, motile, non-phagocytic cells. Antigenic stimulation induces the secretion of lymphokines and leads to changes in the morphology of specific lymphocyte subpopulations. For example, specific binding of antigen to the cell surface stimulates the transformation 10 of small lymphocytes into large ones. Some of these large lymphocytes, of B lineage, differentiate into mature plasma cells, which are active in the synthesis and secretion of immunoglobulin (Ig).

B cells mature in the bone marrow. The earliest 15 identifiable stage of B cell differentiation (in mice) is the pre-B cell, which has immunoglobulin in the cytoplasm but little, if any, on the cell surface. DNA rearrangement is a necessary step for transcription of immunoglobulin genes and, thus, Ig. The presence of 20 cytoplasmic Ig indicates that the DNA that encodes the Ig has already undergone rearrangement by this early stage of differentiation.

Cellular intermediates in the B lymphocyte developmental pathway are distinguished by differences in 25 the organization of the genes that encode the single heavy and light Ig chains, the type of Ig chain (isotype) that is expressed, the amount of Ig that is expressed, and the cell surface antigens that are expressed. B cell maturation is antigen-independent until the mature B cell 30 stage, after which stage, development requires interaction with appropriate mitogens, antigens, T-cell-produced factors, and macrophages. Antigen-stimulated

mature B cells develop into either activated, memory or plasma cells.

For optimum immune responsiveness both B and T cells are necessary. Restoration of immunologic function 5 in animals, whose immune systems have been destroyed by radiation or other treatments, can be achieved by the injection of either bone marrow or thymus cells, however, antibody response is greatest if both cell types can be regenerated.

10 The phenotypes of B, as well as T cells, are determined by the characteristic enzymes or cell surface antigens. B and T cells differ in surface molecules, called differentiation antigens, many of which are "alloantigens", which are encoded by allelic genes and, 15 thus, differ among individuals in the same species. For example, alloantigens can be elicited by injecting mice of a strain that lacks a particular alloantigen with cells from a strain that possess it.

A subpopulation of T-cells, when stimulated by 20 an antigen, produces cell surface molecules that are encoded by a gene locus called the major histocompatibility complex (MHC) in mice. Subsets of T_h cells have surface antigens that are encoded by different portions of the MHC locus. B cells also exhibit cell 25 surface receptors that are encoded by the MHC-locus. MHC-encoded receptors restrict which antigens elicit an immune response by controlling the interaction of T cells with B cells. The specificity of the restriction is determined by the thymic microenvironment (the haplotype 30 of the MHC locus) in which the T cells mature, not by the haplotype of the T cell's MHC locus. The immune response of most lymphocytes depends upon the specific binding of

antigen and the interaction with regulatory lymphocytes and macrophages. The specificity of the T cell response to anti-gen depends, not on recognition of cell-surface antigen alone, but on recognition of the antigen plus 5 products of the MHC locus on the same cell surface. This specificity is called MHC restriction.

Studies of the process of hematopoiesis reveal that numerous hormone-like substances mediate the growth and development of hematopoietic and lymphopoietic 10 progenitor cells and also reveal that the functioning of the immune system involves complex interactions among various cell populations. Lymphokines, which are soluble mediating factors, play a role in communication among the cell populations. For example, T_h cells produce a variety 15 of lymphokines in response to antigenic challenge. The production of lymphokines by these cells is dependent upon the antigen but is independent of T-cell proliferation. T-cell proliferation in response to antigenic challenge represents the proliferation of only 20 a specific subpopulation of T-cells in response to a T_h cell-produced lymphokine.

The lymphokine interleukin-1 (IL-1) facilitates or induces the production of other lymphokines, such as interleukin-2 (IL-2), by T_h cells. IL-2 in turn promotes 25 T-cell proliferation and promotes the differentiation and amplification of certain T-cell subpopulations, including cytotoxic killer cells and tumor infiltrating lymphocytes (TILs). Another such growth substance, erythropoietin, which is induced in vivo by anemia or hypoxia, is 30 required in vitro for the differentiation of erythroid precursor cells (BFU-E) into non-nucleated hemoglobin-producing cells (red blood cells). Granulocyte-macrophage

colony stimulating factor (GM-CSF) stimulates the growth of granulocyte/macrophage colonies, which in turn produce other regulatory proteins.

The processes of proliferation, differentiation and self-renewal are controlled by these specific growth promoting substances or growth regulators. Certain of these growth regulators, which include mitogens, cytokines and lymphokines, are absolutely essential for initiating and sustaining hematopoiesis. Mitogens promote cell division and are responsible for, among other processes, the antigen independent development of lymphoid cells; cytokines are factors, such as lymphokines or monokines, that are produced by cells that affect other cells, and lymphokines are substances that are produced and secreted by activated T lymphocytes and that affect other cell types.

In addition, the presence of growth regulators is absolutely essential for the survival of hematopoietic cells in vitro. Among these regulators, five principle factors that regulate hematopoiesis in vivo, have been identified and produced using recombinant DNA technology: interleukin-3 (IL-3), which stimulates proliferation and development of multipotent stem cells and colony forming cells (CFCs or CFUs) of more restricted potency; granulocyte colony stimulating factor (G-CSF); macrophage colony stimulating factor (M-CSF), which stimulates the development of monocytes and macrophages from GM-CFC; and granulocyte-macrophage colony stimulating factor (GM-CSF). Other factors, such as interleukin-6, which acts on B-cells, and other interleukins and colony stimulating factors have also been produced using recombinant DNA technology.

Clinical trials are presently underway to assess the effectiveness of these factors in promoting hematopoiesis *in vivo* following bone transplantation. In one such trial, recombinant human GM-CSF, which was 5 administered to patients beginning three hours after bone marrow infusion, accelerated myeloid recovery compared to untreated controls (see, Brandt *et al.* (1988) *New Engl. J. Med.* 318: 869-876). GM-CSF has also been shown to stimulate hematopoiesis and induce a fivefold to tenfold 10 increase in circulating blood leukocytes; G-CSF induces an increase in the numbers of stem cells, committed myeloid progenitors, and circulating blood neutrophils; IL-3 stimulates stem cell proliferation *in vivo*; and IL-1 protects mice from potential lethal irradiation without 15 a bone marrow transplant (see, *e.g.* Dexter (1987), supra.). *In vitro* studies are being conducted to evaluate synergistic affects among these factors for accelerating the return of bone marrow function.

Many of the growth promoting substances are 20 essential components of adoptive immunotherapeutic treatments. For example, lymphokines, such as interleukin-2 (IL-2), mediate specific expansion of subpopulations of lymphoid cells that bear specific phenotypic surface markers and that specifically 25 recognize certain antigens on the surfaces of affected cells (see, *e.g.* Knazek *et al.*, supra.). Incubation of resting lymphocytes, which are obtained from tumor bearing hosts, including human and murine hosts, in the presence of IL-2 for three to four days results in the 30 expansion of subpopulations of lymphocytes that are capable of lysing natural killer cell (hereinafter NK)-resistant tumor cells, but not normal cells (see, *e.g.*,

Belldegrun et al. (1989) Chapter 12, in Urologic Oncology, Lepor et al. (eds.), Kluwer Academic Publishers, Boston). This phenomenon is called lymphokine activated killing (hereinafter LAK) and the 5 lymphocytes that are responsible for this phenomenon consist of two types of cells. The first type of cells is called LAK cells and the second type of cells is called TIL cells (see, e.g., Rosenberg (1987a) U.S. Patent No. 4,690,915, which disclosure is herein 10 incorporated in its entirety by reference thereto; see, also, Rosenberg, et al. (1987b) New Eng. J. Med. 316: 889-897, Rosenberg (1986) at pp. 55-91 in Important Advances in Oncology, DeVita et al. (eds.), JB Lippincott, New York, Yron et al. (1980) J. Immunol. 15 125:238, and Rosenberg (1985) Cancer 55: 1327).

TIL cells are lymphocytes that infiltrate into tumors, against which a host's immune system is mounting an immunological response, and can be isolated therefrom (see, e.g., Yron et al., supra.). TIL cells are found 20 to have greater specificity than LAK cells for autologous cells and greater efficacy than LAK cells in adoptive immunotherapy of cancer (see, e.g., Yron et al., supra.). TIL cells have been obtained from resected human tumors, including cancers of the kidney, colon, and breast, 25 melanomas, and sarcomas.

In vitro incubation of cells that have been obtained from a tumor and grown in the presence of IL-2 results in the expansion of activated T cells within the tumor and the destruction of tumor cells or tissue. 30 After 2-3 weeks of culture, the tumor cells have all been destroyed and the culture consists of lymphoid cells that have the phenotype of cytolytic T lymphocytes (CTL) (see,

e.g., Muul et al. (1987a) J. Immunol 138: 989, Topalian et al., supra. and Itoh, et al. (1986) Cancer Res. 46: 3011). Some human TIL cells exhibit a high specificity for their autologous tumors.

5 TIL cells also show promise for use in methods of genetic therapy (see, e.g. Culliton (1989), "News and Comment" in Science 244: 1430-1433 and Knazek et al., supra.). They provide a source of autologous cells that can be modified by the insertions of DNA encoding a
10 desired protein, cultured, and reintroduced into the patient. The desired protein may be a therapeutically effective protein, such as tumor necrosis factor, which is used in cancer therapy, CD4 receptor to which HIV binds, an enzyme, for which the treated host is
15 deficient, or it may be a marker protein, whereby the fate of the TIL cells in the treated host may be studied.

Because many cancer patients do not respond to adoptive immunotherapy, studies are underway to identify other lymphokines, cytokines, and/or mitogens that may be
20 useful alone or in combination with IL-2 in expanding subpopulations of lymphoid cells for use as adoptive immunotherapeutic agents. Although IL-2 has primarily been used to generate such subpopulations of lymphoid cells, other lymphokines, such as IL-4, IL-6 and other
25 interferons, and TNF have also been shown to be useful in the production of in vitro expanded lymphoid cells and may also prove to be useful in expanding specific subpopulations of lymphoid cells. For example, IL-4 (also called BSF-1) is a glycoprotein that is
30 derived from T cells and has been shown to induce LAK activity if the lymphoid cells are first stimulated with IL-2, but is inhibitory if the cells are not pre-

stimulated (Kawakami *et al.* (1989) *J. of Immunol.* 142: 3452-3461) IL-4 also has been shown to be capable of stimulating the growth of TIL cells both alone and in conjunction with IL-2. IL-4 appears to enhance the 5 growth of TIL cells and concomitantly inhibit the growth of NKH1⁺ cells, which are responsible for non-specific killer activity (Lotze *et al.* (1989) at pp. 167-179 in Human Tumor Antigens and Specific Tumor Therapy, Alan R. Liss, Inc., see, also, Kawakami *et al.*, (1988) *J. of Exp. 10 Med.* 168: 2183-2191.).

Disorders of bone marrow cells or bone marrow function and development is implicated in the pathology of numerous diseases, including: leukemias, metastatic cancers, AIDS and other immunodeficiencies, allergies, 15 inherited diseases and others. These diseases, such as leukemia, often result from the depletion, surplus, or absence of certain subpopulations of bone marrow cells or from abnormal cells that develop in certain subpopulations (see, *e.g.* TABLE I, infra.; see, also, 20 *Scientific American Medicine*, Rubenstein and Federman, eds. (April, 1990) Section, 5, Chapter VIII, p. 11). Immune system cell imbalances and defects can arise from defects in the regulation of growth and differentiation of cells in the bone marrow.

25 Many diseases and disorders arise from defects in or alterations in hematopoietic stem cells. Leukemia involves the proliferation of a clone of abnormal hematopoietic cells. Typically leukemic cells exhibit poor responsiveness to normal regulatory mechanisms, a 30 diminished capacity for normal cell differentiation, the ability to expand at the expense of normal myeloid or lymphoid lines, and the ability to suppress or impair

normal myeloid or lymphoid cell growth. Leukemic cells are identified by the particular type of hematopoietic cell that is involved. Thus, myeloid leukemias involve cells derived from myeloid stem cells and lymphoid leukemias involve abnormalities in the cells derived from lymphoid stem cells.

Leukemias are often characterized or identified by typical cytogenetic abnormalities. See, e.g., TABLE I, infra. For example, chronic myelogenous leukemia (CML), which is also called chronic myelocytic leukemia and chronic granulocytic leukemia and which is considered a prototypical stem cell disease (see, e.g., Quesenberry et al. (1979) *New Engl. J. Med.* 301: 868-72), is a clonal disorder. The Philadelphia chromosome, a chromosomal abnormality that is specific for CML, is found in erythroid, granulocytic and megakaryocytic cells lines. That it is found in cells of different lineages indicates that the disease occurs at the pluripotent stem cell level. An abnormal leukemic stem cell arises and gives rise to abnormal red cells, neutrophils, eosinophils, basophils, monocyte-macrophages, platelets, T cells and B cells.

In most cases of CML the Philadelphia chromosome is microscopically visible. The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22. Chromosome 22, which is shortened and usually readily identifiable, is the Philadelphia chromosome. As a result of this translocation, the c-abl oncogene from chromosome 9 is adjacent to the breakpoint cluster region, bcr, gene of chromosome 22. The oncogene and bcr gene encode a chimeric bcr/c-abl mRNA that encodes a tyrosine kinase activity, which is produced in

CML patients who do not appear to have a Philadelphia chromosome. In these patients, the translocation must, however, be present and should be detectable by hybridization of chromosome 22 DNA with probes that span 5 the breakpoint.

Other diseases that are characterized by damaged or deficient pluripotent stem cells include: aplastic anemia, cyclic neutropenia, Blackfan-Diamond syndrome, pure red-cell aplasia, some neutropenias and certain 10 immune deficiency disorders.

Individuals, who are afflicted with a disease of the bone marrow, such as a leukemia, an immunological deficiency, or metastatic cancer, are often treated by bone marrow transplantation. Bone marrow cells are 15 destroyed by gamma or X-ray irradiation. If an animal is irradiated such that only the bone marrow cells are destroyed, the pluripotent stem cells (CFU-S), which occur in the spleen and liver, can repopulate the bone marrow and immune system and the animal does not 20 necessarily die. If the entire body of the animal is irradiated, death is inevitable. It is, however, possible to repopulate and reconstitute the immune system of a potentially lethally irradiated animal by the injection of bone marrow cells. Generally potentially 25 lethal irradiation and/or chemotherapy to destroy the diseased bone marrow cells precedes a bone marrow transplantation. Bone marrow transplantation involves removal of a small amount of bone marrow from the pelvic bone and long bones of the donor and the intravenous 30 introduction of donated marrow into a recipient, who has first been treated with radiation and toxic chemicals to destroy his or her bone marrow and immune cells. Bone

marrow from a matched donor is then injected, and, the pluripotent stem cells within the donor marrow can reconstitute the immune system.

There were approximately 10,000 bone marrow 5 transplantations performed in the United States during 1989. Because of the development of agents that effectively suppress both host rejection of the marrow graft and graft rejection of the host (graft versus host disease) are presently being developed, the therapeutic 10 use of bone marrow transplants is increasing.

There are several general types of bone marrow transplants: autologous transplants in which the patient's own marrow is removed, treated, and returned to the individual and allogenic transplants involving the 15 transplantation of matched marrow from an identical twin, a sibling or an unrelated, but matched, donor.

Autologous bone marrow transplants are performed to reconstitute the marrow of patients who have become severely immunologically deficient secondary to high dose 20 chemotherapy and/or radiation therapy used in treating certain types of cancer, which include some types of lymphomas and testicular and ovarian carcinomas and which, generally do not metastasize to the bone marrow early in the course of the disease. Thus, while 25 chemotherapy/radiation therapy for treating these diseases is expected to destroy the ability of existing marrow to form new cellular components, an aliquot of bone marrow can be removed and saved, prior to treatment of the patient, for subsequent infusion. Typically 400 30 to 800 ml. of marrow is aspirated and frozen until therapy has been completed. The stored marrow is reinfused into the patient, who may, however, remain

immuno-suppressed for several months until his or her immune system becomes reconstituted.

Allogenic transplants, which pose substantially greater risk to the patient, are used when an autologous transplant cannot be used or is unavailable, such as in cases of patients who have genetic defects or metastatic spread of a malignancy to the bone marrow. The donor is matched closely to the human leukocyte antigen (HLA) phenotype. Human leukocyte antigens are encoded by the major histocompatibility complex genes, which are analogous to the MHC locus in mice, and are present on the cell surfaces. Matching the antigens of the donor with those of the recipient lessens the likelihood of host and/or graft rejection. The closer the HLA match 15 the greater the likelihood of engraftment. The likelihood of engraftment of slightly mismatched transplants can be increased by incubating the donor marrow in the presence of anti-T cell antibody, which destroys these mediators of rejection. The allogenic 20 recipient is commonly treated with agents, such as cyclosporin, to suppress the rejection.

Because of the difficulties in finding matched donors, treatments in which autologous transplants can be used are the most promising. Such treatments may involve 25 removal of some bone marrow prior to irradiation and then treatment of that bone marrow aliquot with agents that preferentially destroy the diseased cells. After irradiation of the afflicted individual, the treated bone marrow is reinfused to repopulate the hematopoietic and 30 immune systems with healthy cells. These treatments can be used, for example, for treating cancer and potentially lethally irradiated victims of nuclear accidents. This

method has been used to treat individuals suffering from metastatic breast cancer, which typically metastasizes to the bone marrow.

- After transplantation, the donor marrow must
- 5 reconstitute the immune system of the recipient. The process of marrow engraftment and hematopoietic reconstitution takes nearly three weeks and complete regeneration of the immune system can take many months. Prior to hematopoietic reconstitution, the recipient is
- 10 at risk of contracting infections, other diseases, and, except for autologous or allogenic transplants from an identical twin, rejecting the donated marrow. Currently, it is often difficult to obtain sufficient amounts of bone marrow for transplantation and for in
- 15 vitro manipulation. For successful transplantation, not only must the marrow be matched to the recipient, it must contain a sufficient number of pluripotent stem cells to reconstitute the immune system of the recipient sufficiently fast before the recipient succumbs to
- 20 infection. Once marrow is removed, it is difficult, if not impossible, to culture it under conditions whereby pluripotent stem cells proliferate. Generally, any manipulation causes the pluripotent stem cells to irreversibly commit to a particular lineage.
- 25 In vitro bone marrow cultures.

- Among the goals of in vitro bone marrow cell culture is development of means to maintain such cultures indefinitely, and perhaps, more important, to develop a culture system whereby pluripotent stem cells can be maintained without differentiating into a committed pathway so that such cells could be cultured and then used to repopulate an irradiated or otherwise damaged

immune system. In attempting to develop immortal cultures efforts have been made to mimic the events that occur in vivo. Numerous bone marrow culture systems have been developed, but thus far, none satisfy 5 these criteria.

In 1966 Bradley and Metcalf (see, e.g., Aust. J. Exp. Biol. Med. Sci. 44: 287) and Pluznick and Sachs (see, e.g., Exp. Cell Res. 43: 553) independently reported that mouse bone marrow cells would form colonies 10 of granulocytes and macrophages when plated in soft agar, which contains horse serum, Fischer's salts, and an appropriate source of GM-CSF. In vitro colony formation was found to absolutely require a continuous source of this CSF. Medium conditioned by certain cell 15 types, such as heart or lung, were found to serve as a source of CSF.

In the agar culture system, the number of colonies that develop from a given concentration of hematopoietic cells depends upon the concentration of CSF 20 (high concentrations inhibit colony formation and low concentrations are insufficient) and upon the quality (lot) of horse serum used. In this system hematopoietic cells proliferate for only a week to ten days and differentiation to committed hematopoietic progenitors 25 (GM-CFUs) continues for 2 to 3 weeks (see, e.g., Dexter et al. (1976) Methods in Cell Biol. 14: 387). Thus, this system has not proven to be satisfactory because sustained hematopoiesis cannot be maintained. Colony formation in agar or other semi-solid culture 30 medium, however, has been used to assay for the concentrations of various committed progenitor cells in an aliquot of bone marrow cells, since colony formation

is proportional to the number of committed cells. The type of colony formed is a function of the type of CSF activity that is added to the agar.

Attempts to sustain continuous and normal hematopoiesis in vitro have been more successful using liquid culture systems than solid systems. The first liquid systems developed were of two types: co-cultures of bone marrow cells plus thymus cells (see, e.g., Dexter, et al. (1973) *J. Cell. Phys.* 82: 461) and cultures in which bone marrow cells are added to an established bone marrow culture, bone marrow plus bone marrow cultures (see, e.g. Dexter et al. (1976) *Meth. Cell Biol.* 14: 387). The latter appear to more accurately mimic in vivo hematopoiesis.

As discussed above, the thymus is known to influence hematopoiesis of bone marrow cells. It has been shown that incubation of bone marrow cells with either thymocytes (see, Miller (1973) *J. Immunol.* 111: 1005) or thymic factors (see, Miller (1974) *J. Immunol.* 113: 110) in 20% fetal calf serum (FCS), minimal essential medium (MEM), vitamins, non-essential amino acids, and antibiotics produces functional T cells that are able to "help" B cells in exhibiting an anti-sheep red blood cell response in vitro.

This method for maintaining stem cell proliferation and hematopoiesis in culture was the first somewhat successful method. Suspensions of thymus cells are incubated in Dexter medium (supra.) in glass culture bottles or flasks. After several days the cultures consist of a population of cells that adhere to the glass surfaces and a population of cells in the overlying medium, which is decanted. The adherent cells are a

mixture of cell types, including fibroblastic, epithelioid and phagocytic cells.

After the overlying cells are decanted, bone marrow cells are then added to the cultures.

5 Initially the overlying bone marrow cells are primarily granulocytes in various stages of maturation. After two weeks, however, the culture becomes either one that produces primarily granulocytes (G-type) or one that produces primarily macrophages (M-type). Commitment to
10 either type occurs during the first week in culture. In the M-type cultures the number of CFU-S in the overlying medium decline and are gone by week five, GM-CFU decline in number and are gone by week 7 and granulocytes disappear. In the G-type cultures CFU-S persist for over
15 12 weeks, the percentage of mononuclear phagocytic cell's decreases over time, and GM-CFU persist for at least 10 weeks.

The bone marrow plus bone marrow liquid culture method, which uses multiple inoculations of bone marrow
20 into a liquid culture, was first described by Dexter and Lajtha in 1974 (see, Br. J. Haematol. 28: 525). Pooled murine femoral bone marrow cells are inoculated at a concentration of 10^6 nucleated cells per milliliter into non-siliconized tissue culture bottles that contain
25 Fischer's medium and 20% horse serum. At first the success of the culture was dependent upon the lot of horse serum used, but later (see, e.g., Greenberger et al. (1979) J. Exp. Haematol. 7: Supp. 5: 135) it was discovered by adding of hydrocortisone (final
30 concentration about 10^{-7} M) to the medium the dependence upon the particular lot of horse serum was eliminated and any lot of horse or fetal calf serum could be used to

maintain the cultures.

After the first inoculation of bone marrow cells, a few cells adhere to the glass and some develop fat vacuoles. Stem cells die rapidly. After one week, 5 and thereafter once a week, the bottles are gently shaken and half of the growth medium is removed and replaced with fresh medium. After three weeks the GM-CFU and CFU-S have substantially declined in number and an adherent layer has formed on the surface bottles. The 10 adherent layer appears to provide an appropriate microenvironment for sustained hematopoiesis (see, e.g., Dexter (1982), supra). The cultures are then inoculated a second time with bone marrow cells after which the suspended cells and half to three-fourths of the 15 suspended medium is removed once a week and replaced with fresh medium. This technique has been reported to produce cultures that generate pluripotent CFU-S and GM-CFU for up to one year (see, e.g., (1979) Greenberger et al. (1979) *Virol.* 95: 317).

20 These long-term cultures contain pluripotent stem cells, granulocyte and macrophage committed progenitors, erythroid progenitors and lymphoid progenitors. Committed hematopoietic progenitors are continuously released into the overlying growth medium. 25 Eventually, however, the cultures become predominantly populated with phagocytic mononuclear cells (see, e.g. Dexter, et al. (1980) *J. Supramol. Struct.* 13: 513).

30 To some extent these long-term cultures mimic in vivo hematopoiesis in the mouse (Dexter, et al. (1980), supra). For every CFU-S in culture about ten GM-CFU are produced and for every GM-CFU about 500-1000 mature granulocytes develop, which approximate their respective

in vivo proportions. The mature granulocytes appear to be identical to their in vivo counterparts. These cultures also produce mature megakaryocytes (platelet producers) and erythroid precursors. Erythropoiesis is 5 blocked at the 10-14 day BFU-E stage. The BFU-E mature, if erythropoietin is added to the culture medium, but hemoglobin is not produced. If, however, the cultures are gently shaken during erythropoietin treatment, mature non-nucleated hemoglobin-containing cells are produced 10 (see, Dexter *et al.* (1981) *Blood* 58: 699).

The adherent layer is composed of several phenotypically distinguishable cell types, including endothelial-appearing cells, adipocytes, and reticular cells (see, *e.g.* Hines (1983) *Blood* 61: 397). It forms 15 during the first three weeks of culture, before the second inoculation. Within two to three weeks after inoculation, the adherent layer appears to consist of a multi-layer pavement-like structure of endothelial cells, large branching dendritic cells, foci of lipid-filled 20 adipocytes and some macrophages. CFU-S, present in the second inoculum, migrate to this layer and form membrane-functional complexes with cells in the adherent layer. Committed progenitors and mature hematopoietic cells are continuously released into the overlying medium (see, 25 Dexter (1982) *J. Cell. Phys. Supp.* 1: 87).

Close range cell-to-cell interactions between the overlying cells and adherent layer appear to be necessary for sustained hematopoiesis to occur. If diffusion chambers are placed on pre-formed layers, 30 hematopoiesis is not maintained (see, Bentley (1981) *Exp. Haematol.* 8: 77). Also, maintenance of the Dexter long-term bone marrow cultures is dependent upon the

formation of the adherent layer from the first marrow inoculum. In the absence of the adherent layer, sustained hematopoiesis does not occur. Adherent layers formed from tissues other than bone marrow, such as 5 spleen or thymus, do not sustain active hematopoiesis (see, Dexter (1980) *J. Supramol. Struct.* 13: 510). Adherent layers from other tissues do not develop adipocytes, which may, therefore, be responsible or contribute to the sustenance of hematopoiesis. It is the 10 addition of hydrocortisone the medium that induces differentiation of pre-adipocytes into adipocytes. As discussed above, hydrocortisone or an appropriate lot of horse serum is needed to achieve sustained hematopoiesis. It has also been found (see, Greenberger et al. (1979), 15 supra.) that infection of Dexter cultures with murine sarcoma virus, which infects pre-adipocytes, blocks hematopoiesis. Thus, it appears that the adipocytes play an important role in sustaining hematopoiesis.

Thus, the adherent layer appears to produce at 20 least some factors that participate in sustaining hematopoiesis. The rate of proliferation of CFU-S cycles over time. This cycling appears to be associated with the production of molecules in the adherent layer that either stimulates or inhibit DNA synthesis in CFU-S. The 25 concentration of stimulatory material increases relative to the concentration of the inhibitory material shortly after the cells are fed. Several days later the relative concentrations are reversed. The cycling can be altered by adding either factor to the medium.

30 When injected into lethally irradiated mice, long term Dexter cultures reconstitute the immune system (see, Schrader et al. (1978), supra.). In addition, the

B cell progenitors and Thy1⁺ cytotoxic cells are specifically expanded if an aliquot of the culture is transferred to lymphocyte-conditioned medium (see, Dorshkine et al. (1982) J. Immunol. 129: 2444). The 5 Dexter culture system microenvironment does not, however, produce functional mature T-lymphoid cells.

It has also been possible to culture non-adherent murine bone marrow cells in the absence of an adherent layer by transferring them to medium 10 supplemented with a dialyzable "factor" produced by the murine myelomonocytic leukemic cell line WEHI-3b. The self-renewal capacity of multipotent stem cells and cloned sublines of GM-CFUs can be maintained for up to two years when grown in this medium (see, Greenberger et 15 al. (1981), supra.).

The Dexter method for long term culture of murine bone marrow cells, however, fails to achieve sustained hematopoiesis of human bone marrow specimens. It is found that cultures of human bone marrow cells 20 steadily decline in viability (see, e.g., Greenberger et al. (1979) Exp. Haematol. 7 (Supp. 5): 135). In human bone marrow cultures the adherent layer does not develop properly. It develops very slowly, few foci of adipocytes are observed, it becomes overgrown with 25 fibroblasts and activated macrophages, and the cells tend to pile up rather than spread out, leading to necrosis and detachment of the cells (see, e.g., Moore, (1979) Blood 54: 77; (1980) Blood 55: 687; and Hocking et al. (1980) blood 61: 770).

30 Rather than the absence of a proper adherent layer in human bone marrow cultures, it may be the high level of endogenous GM-CSF that accounts for the

- inability to sustain hematopoiesis (see, e.g. Moore (1980) supra). The high concentration of endogenous CSF rapidly induces conversion of cultures to macrophages with a concomitant loss of CFU-S production. If a 5 culture rapidly becomes committed to an end-stage cell, sustained hematopoiesis is impossible. Additionally, unlike in murine bone marrow cultures, in human bone marrow cultures lymphocytes, particularly T cells, persist.
- 10 Modification of the Dexter method, has, however, produced human bone marrow cultures that have been maintained for as long as 20 weeks (see, Greenberger et al. (1981) Blood 58: 724, Moore et al. (1980) Blood 55: 682). These modifications include: growth at 37° C, 15 rather than 33° C, which is optimal for murine cultures, and elimination of the second inoculum of bone marrow cells.

1 The ability to grow bone marrow cells in vitro makes it possible to, not only study the growth and 2 regulation of bone marrow cells, but to use such cells 3 for transplantation. If means to treat diseased bone 4 marrow cells and sustain the growth of the healthy 5 pluripotent cells in vitro could be devised, it would be 6 of immense importance for the treatment of leukemias, 7 metastatic cancers, particularly breast cancer, AIDS, and 8 other immune system disorders.

9 There is, thus, a need for the development of 10 methods that can be used to efficiently and cost 11 effectively culture human bone marrow cells in a manner 12 that does not result in rapid commitment to end-stage 13 cells, but rather that maintains or increases the numbers 14 of pluripotent stem cells present in the initial

1 inoculum. Development of such methods will provide a
 2 means for genetically engineering pluripotent stem cells
 3 for use in methods of genetic therapy.

TABLE I

<u>Karyotypic abnormality</u>	<u>Clinical Findings</u>
6 t(8;21)	Acute myeloblastic leukemia
7 t(15;17)	Acute promyelocytic leukemia
8 t/del(11)	Acute myeloblastic or acute 9 monocytic leukemia
10 inv/del(16)	Acute myelomonocytic leukemia
11 t(9;22)	Chronic myeloid leukemia 12 Acute myeloblastic leukemia 13 (rare) 14 Acute lymphocytic leukemia
15 t(6;9)	Acute myeloblastic or acute 16 myelomonocytic leukemia
17 inv(3)	Acute myeloblastic leukemia
18 trisomy 8	Acute myeloblastic leukemia 19 Myelodysplastic syndrome
20 loss of chromosome 21 5 or 7	Acute myeloblastic leukemia Myelodysplastic syndrome
22 5q	Acute myeloblastic leukemia Myelodysplastic syndrome
24 t(8;14), t(2;8), or 25 t(8;22)	Acute lymphocytic leukemia Burkitt's leukemia
26 trisomy 12	Chronic lymphocytic leukemia
27	see, Scientific American Medicine, Rubenstein and
28 Federman, eds. (April, 1990) Section, 5, Chapter VIII, p.	29 11.
30	

1 SUMMARY OF THE INVENTION
2

3 It is one object of this invention to provide an
4 improved method for culturing bone marrow cells,
5 comprising: (a) inoculating the extra fiber space of a
6 hollow fiber bioreactor that is a component of a hollow
7 fiber culture system with a suspension of bone marrow;
8 and (b) incubating said suspension in said bioreactor,
9 whereby said at least a portion of the pluripotent stem
cells in said suspension proliferate or are maintained.

10 It is another object of this invention to
11 provide a method of adoptive immunotherapy for the
12 treatment of cancer, comprising: (a) obtaining from a
13 donor bone marrow that contains at least an effective
14 number of pluripotent stem cells; (b) inoculating the
15 extra fiber space of a hollow fiber bioreactor that is a
16 component of a hollow fiber culture system with a
17 suspension of said bone marrow cells; and (c) incubating
18 said cells in said bioreactor under conditions
19 in which said cells remain viable, whereby at least some
20 of the pluripotent stem cells of said bone marrow cells
21 proliferate or retain the ability to differentiate,
22 wherein said effective number is capable of
23 reconstituting the immune system of a recipient of said
24 cells after said cells have been cultured.

25 It is another object of this invention to
26 provide a method of adoptive immunotherapy for the
27 treatment of neoplastic disease, comprising: (a)
28 obtaining from an individual afflicted with a neoplastic
29 disease bone marrow that contains at least an effective
30 number of pluripotent stem cells, wherein the individual
31 is subsequently treated with chemotherapy and/or
32 radiation therapy to destroy or inactivate neoplastic

1 cells; (b) inoculating the extra fiber space of a hollow
2 fiber bioreactor that is a component of a hollow fiber
3 culture system with a suspension of the bone marrow; and
4 (c) incubating said cells in said bioreactor under
5 conditions in which said cells remain viable, whereby at
6 least some of the pluripotent stem cells of said bone
7 marrow cells proliferate or retain the ability to
8 differentiate, wherein said effective number is capable
9 of reconstituting the immune system of the individual
10 after said cells have been cultured.

11 It is another object of this invention to
12 provide to provide a method of adoptive immunotherapy for
13 the treatment of neoplastic disease, comprising: (a)
14 obtaining from an individual afflicted with a neoplastic
15 disease bone marrow that contains at least an effective
16 number of pluripotent stem cells, wherein the individual
17 is subsequently treated with chemotherapy and/or
18 radiation therapy to destroy or inactivate neoplastic
19 cells; (b) inoculating the extra fiber space of a hollow
20 fiber bioreactor that is a component of a hollow fiber
21 culture system with a suspension of the bone marrow and
22 adding an effective amount of at least one growth
23 promoting substance that specifically expands a
24 therapeutically useful subpopulation of lymphoid cells is
25 added to the extra fiber space (EFS) of said bioreactor,
26 wherein said effective amount is an amount sufficient to
27 effect said specific expansion and said subpopulation is
28 effective in inactivating said neoplastic cells; and (c)
29 incubating said cells in said bioreactor under conditions
30 in which said cells remain viable, whereby at least some
31 of the pluripotent stem cells of said bone marrow cells
32 proliferate or retain the ability to differentiate,

1 wherein said effective number is capable of
2 reconstituting the immune system of the individual after
3 said cells have been cultured.

4 It is another object of this invention to
5 provide a method for clearing neoplastic cells from bone
6 marrow, comprising culturing bone marrow cells that
7 contain said neoplastic cells in a bioreactor.

8 It is another object of this invention to
9 provide a method for preparing bone marrow cell
10 conditioned medium for use in stimulating the growth of
11 cells and as a source of biologically active growth
12 promoting substances, comprising removing the contents of
13 the extra-fiber space of a bioreactor in which bone
14 marrow cells have been cultured, pelleting and removing
15 the cells from said contents of the extra fiber space to
16 produce an extra fiber space cell supernatant; and
17 dialyzing said extra fiber space cell supernatant against
18 tissue culture medium to produce extra fiber space
19 conditioned medium.

20 This invention significantly improves the
21 procedure for culturing bone marrow cells in vitro by
22 providing an improved method for culturing said cells
23 that can be adapted to methods in which bone marrow cells
24 may be cleared of diseased cells or modified by
25 introduction of heterologous DNA.

26 In practicing this invention therapeutically
27 useful yields of biologically active bone marrow cells
28 that contain a proportion of pluripotent stem cells that
29 is at least as high as that in the inoculum are
30 obtained.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a scheme of the pathways by which stem cells differentiate and develop into the various lineage restricted cell types, which are of limited potency, from which the end state differentiated cells, which lack or are of severely restricted potency, develop (see, Dexter (1987) *supra*).

Figure 2 is a scanning electron micrograph of normal bone marrow cells growing in between the hollow fibers of the CELLMAX™ 100 bioreactor.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, adoptive immunotherapy is a therapeutic method, whereby cells of the immune system are removed from an individual, cultured and/or manipulated *in vitro*, and introduced into the same or a different individual as part of a therapeutic treatment for an acquired or inherited disease.

As used herein, bone marrow includes any cells that are derived from or are part of the bone marrow and also includes other substances derived from or components of the bone marrow. Included among such cells and substances are all hematopoietic and lymphoid cell progenitors, including pluripotent stem cells, stromal cells, which include adipocytes, fibroblasts and endothelial cells, and the bone marrow extracellular matrix, which includes laminin, collagen, and glycosaminoglycans to which some growth factors that are produced by stromal cells and hematopoietic cells bind. If the bone marrow cells are derived from an individual suffering from leukemia or other cancer, bone marrow cells include any leukemic or other cancerous cell present in the bone marrow.

1 As used herein, culturing of bone marrow cells
2 refers to the introduction of bone marrow cells into a
3 suitable medium at an appropriate temperature, generally
4 about 32-37° C, in suitable tissue culture medium and the
5 maintenance or increase in the relative proportion of
6 pluripotent stem cells under these conditions in vitro
7 for periods of time of days up to months.

8 In particular, bone marrow cells are cultured
9 in vitro in order to provide a source of healthy
10 pluripotent stem cells that are used in methods of
11 treatment that require bone marrow transplantation. The
12 bone marrow cells that are produced in accordance with
13 the methods disclosed of this invention are herein
14 referred to as in vitro cultured bone marrow cells.

15 The bone marrow cells may be cultured in the
16 presence of chemical agents, other cells, such as TIL
17 cells, or in the presence of growth promoting substances
18 that expand particular subpopulations of the bone marrow
19 cells, including TIL cells. If the bone marrow cells are
20 cultured in the presence of a cytokine, such as IL-2,
21 then the in vitro expanded subpopulations of cells that
22 are produced include activated lymphoid cells and,
23 depending upon the source thereof and the cytokine used,
24 may include LAK cells and TIL cells. Such growth
25 promoting substances include, but are not limited to
26 cytokines, such as IL-2, IL-1, IL-6 and IL-4 or mixtures
27 thereof. If the bone marrow cells that are expanded in
28 the presence of the cytokine are derived from a patient
29 suffering from a tumor that has metastasized to the bone
30 marrow, then the in vitro expanded subpopulation of cells
31 that is produced may include CTL, LAK, and/or TIL cells.

1 Bone marrow cells may also be genetically
2 engineered to express heterologous gene products by
3 culturing such cells in the presence of an effective
4 concentration of a recombinant vector or recombinant
5 viral vector, whereby heterologous DNA included in the
6 vector becomes stably incorporated into the bone marrow
7 cells and the products expressed by the bone marrow
8 cells, particularly pluripotent or multipotent stem
9 cells.

10 As used herein, neoplastic cells include any
11 type of transformed or altered cell that exhibits
12 characteristics typical of transformed cells, such as a
13 lack of contact inhibition and the acquisition of tumor-
14 specific antigens. Such cells include, but are not
15 limited to leukemic cells and cells derived from a tumor.

16 As used herein, neoplastic disease is any
17 disease in which neoplastic cells are present in the
18 individual afflicted with the disease. Such diseases
19 include, any disease characterized as cancer.

20 As used herein, heterologous DNA is DNA that
21 encodes proteins that are not normally produced in vivo
22 by the cells. Examples of such proteins include
23 traceable foreign marker proteins, such as a protein that
24 confers neomycin resistance, and therapeutically
25 effective substances, such as anti-cancer agents. Cells
26 may be genetically engineered to contain and to express
27 DNA encoding drug resistance or drug sensitivity, such as
28 methotrexate resistance, so that, when such DNA is
29 expressed, such cells may be selectively expanded or
30 destroyed in vivo. In addition, genetic therapy may be
31 used to correct genetic disorders. The cells of an
32 individual who suffers from an inherited or acquired

1 genetic defect, such as β -thalassemia, may be genetically
2 engineered to correct the defect by incorporation of DNA
3 that encodes a normal version of the defective gene.

4 As used herein, lymphoid cells include
5 lymphocytes, macrophages, and monocytes that are derived
6 from any tissue in which such cells are present. In
7 general lymphoid cells are removed from an individual who
8 is to be treated. The lymphoid cells may be derived from
9 a tumor, peripheral blood, or other tissues, such as the
10 lymph nodes and spleen that contain or produce lymphoid
11 cells.

12 As used herein, therapeutically useful
13 subpopulations of in vitro expanded bone marrow or
14 lymphoid cells are cells that are expanded upon exposure
15 of bone marrow or lymphoid cells to a growth promoting
16 substances, such as lymphokines, when bone marrow or
17 lymphoid cells are cultured in vitro. For example,
18 culturing bone marrow cells in the presence of IL-2,
19 preferentially expands lymphocyte subpopulations present
20 in then inoculum.

21 As used herein, a target antigen is an antigen
22 that is present on the surface of a cancerous cell that
23 is specifically recognized by a subpopulation of in vitro
24 expanded lymphoid cells. Such cancerous cells may be
25 found in the bone marrow of patients suffering from
26 metastatic tumors.

27 As used herein, tumor-specific in vitro expanded
28 lymphoid cells are cells that specifically recognize
29 target antigens that are present on or in tumor cells.
30 TIL cells are tumor specific lymphoid cells. As used
31 herein a tumor-specific antigen is an antigen that is
32 disposed on the surface or inside of a tumor cell. Tumor

1 specific antigens may be used in purified form, on
2 irradiated tumor cells, or they may be obtained by
3 purifying them from tumor cells or by synthesizing them
4 in vitro by methods, such as genetic engineering.

5 As used herein, a growth promoting substance is
6 a substance, that may be soluble or insoluble, that
7 in some manner participates in or induces cells to
8 differentiate, activate, grow and/or divide. Growth
9 promoting substances include mitogens and cytokines.
10 Examples of growth promoting substances include the
11 fibroblast growth factors, osteogenin, which has been
12 purified from demineralized bone (see, Luyten, F. P. et
13 al. (1989) *J. Biol. Chem.* 264: 13377), epidermal growth
14 factor, the products of oncogenes, the interleukins,
15 colony stimulating factors, and any other of such factors
16 that are known to those of skill in the art.
17 Recombinantly-produced growth promoting substances, such
18 as recombinantly-produced interleukins, are suitable for
19 use in this invention. Means to clone DNA encoding such
20 proteins and the means to produce biologically active
21 proteins from such cloned DNA are within the skill in the
22 art. For example, interleukins 1 through 6 have been
23 cloned. Various growth promoting substances and
24 combinations thereof may be used to expand desired
25 subpopulations of lymphoid cells.

26 As used herein, a mitogen is a substance that
27 induces cells to divide and in particular, as used
28 herein, are substances that stimulate a lymphocyte
29 population in an antigen-independent manner to
30 proliferate and differentiate into effector cells.
31 Examples of such substances include lectins and
32 lipopolysaccharides.

1 As used herein, a cytokine is a factor, such as
2 lymphokine or monokine, that is produced by cells that
3 affect the same or other cells.

4 As used herein, a lymphokine is a substance that
5 is produced and secreted by activated T lymphocytes and
6 that affects the same or other cell types. Tumor
7 necrosis factor, the interleukins and the interferons are
8 examples of lymphokines. A monokine is a substance that
9 is secreted by monocytes or macrophages that affects the
10 same or other cells.

11 As used herein, an effective number of in vitro
12 expanded lymphoid cells is the number of such cells that
13 is at least sufficient to achieve a desired therapeutic
14 effect, when such cells are used in a particular method
15 of adoptive immunotherapy. For example, an effective
16 number of TILs may be added to a bone marrow culture in
17 a bioreactor or mixed with the bone marrow cells prior to
18 or upon inoculation into a bioreactor in order to clear
19 the bone marrow of all cancerous cells.

20 As used herein, an effective amount of growth
21 promoting substance is an amount that is effective
22 in inducing a particular subpopulation of bone marrow
23 cells. For example, an effective amount of IL-2 may be
24 an amount that is effective in inducing activation
25 and/or proliferation of TIL cells in the bone marrow
26 inoculum, whereby all cancerous cells in the bone marrow
27 are inactivated.

28 As used herein, a hollow cell fiber culture
29 system consists of a hollow fiber bioreactor as well as
30 pumping means for perfusing medium through said system,
31 reservoir means for providing and collecting medium, and
32 other components, including electronic controlling,

1 recording or sensing devices. A hollow fiber bioreactor
2 is a cartridge that consists of a multitude of semi-
3 permeable tube-shaped fibers encased in a hollow shell.

4 The terms hollow fiber reactor and hollow fiber
5 bioreactor are used interchangeably. For example, a "B3"
6 bioreactor cartridge is one that contains a plurality of
7 fibers consist of semi-permeable DEAE-cellulose fibers
8 that have a nominal molecular weight cut-off of about
9 3,000 Daltons. A "B4" cartridge in which the DEAE-
10 cellulose fibers have a nominal molecular weight cut-off
11 of about 4,000 Daltons. A type "A" cartridge includes
12 fibers that are constructed of polyolefin and whose fiber
13 walls have pores of about 0.5 microns diameter.

14 As used herein, the extra fiber space (EFS) is
15 the space in which the cells grow within the shell of the
16 hollow fiber bioreactor that is external to the semi-
17 permeable fibers. The EFS is alternatively referred to
18 as the extra capillary space (ECS).

19 As used herein, the EFS cell supernatant is the
20 medium in which the cells in the EFS are growing. It
21 contains secreted cellular products, diffusible nutrients
22 and any growth promoting or suppressing substances, such
23 as lymphokines and cytokines, produced by the cultured
24 bone marrow cells or added to the EFS or tissue culture
25 medium. The particular components included in the EFS is
26 a function not only of what is inoculated therein, but
27 also of the characteristics of the selected hollow fiber.

28 Thus, as used herein, a hollow fiber bioreactor
29 or hollow fiber bioreactor cartridge consists of an outer
30 shell casing that is suitable for the growth of mammalian
31 cells, a plurality of semi-permeable hollow fibers
32 encased within the shell that are suitable for the growth

1 of mammalian cells on or near them, and the EFS, which
2 contains the cells and the EFS cell supernatant.

3 Tissue culture medium perfuses through the fiber
4 lumens and is also included within the shell surrounding
5 said fibers. The tissue culture medium, which may differ
6 in these two compartments, contains diffusible components
7 that are capable of sustaining and permitting
8 proliferation of any CFU-S in the bone marrow cells.
9 The medium is provided in a reservoir from which it is
10 pumped through the fibers. The flow rate can be
11 controlled varied by the varying the applied pressure.

12 The EFS or perfusing medium may additionally
13 contain an effective amount of at least one growth
14 promoting or suppressing substance that specifically
15 promotes the expansion or suppression of at least one
16 subpopulation of the bone marrow cells, such as TIL
17 cells, in which the effective amount is an amount
18 sufficient to effect said specific expansion.

19 As used herein, EFS conditioned medium is the
20 EFS cell supernatant after it has been centrifuged to
21 remove any cells and particulate matter and dialyzed
22 against tissue culture medium.

23 As used herein, tissue culture medium includes
24 any culture medium that is suitable for the growth of
25 mammalian cells and in which bone marrow cells remain
26 viable in vitro. Examples of such medium include, but
27 are not limited to AIM-V and Iscove's medium (GIBCO,
28 Grand Island, N.Y.).

29 The medium may be supplemented with additional
30 ingredients including serum, serum proteins, growth
31 suppressing, and growth promoting substances, such as
32 cytokines, and selective agents for selecting genetically

1 engineered or modified cells.

2 As used herein, complete AIM-V is a tissue
3 culture medium that consists of the proprietary formula
4 AIM-V (GIBCO, Grand Island, N.Y.) and also contains 10
5 μ g. gentamicin/ml. (GIBCO), 50 μ g. streptomycin/ml.
6 (GIBCO), 50 μ g penicillin/ml. (GIBCO), 1.25 μ g.
7 fungizone/ml. (Flow Laboratories, MacLean, VA.).

8 As used herein, AIM-V supernatant is prepared as
9 described in Muul et al. (1986) J. Immunol. Methods 88:
10 265). Briefly, LAK AIM-V supernatant is prepared by
11 growing peripheral blood lymphocytes in AIM-V or other
12 suitable tissue culture medium in the presence of IL-2
13 for 2 to 3 days and removing the cells by centrifugation
14 to obtain the supernatant.

15 Other suitable tissue culture media are well-
16 known and readily available to those of skill in the art
17 and may be readily substituted for AIM-V. For example,
18 a medium that consists of a 50-50 mixture of complete
19 AIM-V and RPMI having 10% heat-inactivated human serum,
20 and further supplemented with LAK supernatant may be
21 used.

22 Hollow fiber bioreactors (abbreviated herein as
23 HF) are known to those of skill in the art (see, e.g.,
24 Knazek et al., U.S. Patent Nos. 4,220,725, 4,206,015,
25 4,200,689, 3,883,393, and 3,821,087, which disclosures
26 are herein incorporated by reference thereto). Hollow
27 fiber bioreactors have been used for the growth of
28 mammalian cells and for the production of biologically
29 active products that are secreted thereby (see, e.g.,
30 Knazek et al. supra., see, also, Yoshida et al. U.S.
31 Patent No. 4,391,912; Meyers et al. U.S. Patent No.
32 4,546,083; and Markus et al., U.S. Patent No. 4,301,249).

1 Hollow fiber bioreactors have, not however, heretofore
2 been used for the selective growth of biologically active
3 cells, such as the in vitro expanded lymphoid cells of
4 this invention, which cells are used in vivo in methods
5 of adoptive immunotherapy.

6 The hollow fiber bioreactor that is contemplated
7 for use in the practicing this invention contains a
8 multitude of tube shaped semi-permeable membranes
9 (hereinafter called fibers) that are encased in a hollow
10 shell. Cultured cells grow and fill the spaces between
11 the fibers and are fed by passage of nutrients through
12 the fiber walls from medium that is perfuses through the
13 lumina of said membranes. An example of a hollow fiber
14 bioreactor that may be used in practicing this invention
15 is the hollow fiber bioreactor, B3, Cellco Advanced
16 Bioreactors, Inc., Kensington, MD, or the hollow fiber
17 bioreactor, B4, Cellco Advanced Bioreactors, Inc.,
18 Kensington, MD, (see U.S. Application Serial (see U.S.
19 Application Serial No. 07/238,445, supra for a complete
20 description thereof). The bioreactor, B3, contains
21 about 6000 tube-shaped, semi-permeable membranes, which
22 provide a 1.1 m^2 surface area. The fibers, which are each
23 approximately $250 \mu\text{m}$ in diameter, are pulled through a
24 polycarbonate tube that is about 12 inches in length, and
25 sealed at each end in such a manner that liquid only
26 flows through the lumina of the fibers to exit at the
27 opposite end of the shell. The fiber walls nominally
28 restrict passage to substances having molecular weights
29 less than a desired cut-off range. The fibers divide the
30 cartridge into the extra-fiber space (EFS), typically
31 about 50 ml. in volume, and the volume within the fiber
32 lumina. The fibers and shell form a hollow fiber

1 cartridge. Minimal bulk flow of liquid occurs within the
2 extra-fiber space, which is also referred to as the
3 extra-capillary or shell-side space.

4 If desired, prior to use, growth promoting
5 substances or vectors may be bound to the fibers,
6 introduced into the EFS, or included in the perfusing
7 medium. The fibers are selected as a function of the
8 components of the perfusing medium to which they must be
9 permeable and as a function of the components of the EFS.
10 For example, if desired, the fibers may be selected so
11 that exogenous growth promoting substances can bind
12 thereto. Binding may be irreversible and may be
13 accomplished by the use of cross-linking agents, such as
14 glycosaminoglycans, or other methods known to those of
15 skill in the art or binding may be reversible, such as by
16 absorption of the antigen or substance to the fiber.
17 Glycosaminoglycans, to which colony stimulating factors
18 and other growth factors bind in vivo (see, e.g., Gordon
19 et al. (1987) *Nature* 326: 403-405), may be bound to the
20 fibers or to stromal cells in the bioreactor. Both GM-
21 CSF and IL-3 specifically bind to the glycosaminoglycan,
22 heparin sulfate, which is a part of the bone marrow
23 stromal extracellular matrix. The growth factor, which
24 is then added to the bioreactor, binds to the
25 glycosaminoglycans, assumes an active conformation by
26 virtue of this binding, and thereby mimics its in vivo
27 activity. Alternatively, glycosaminoglycans can be added
28 to the bioreactor with stromal cells. Growth promoting
29 substances, including colony stimulating factors,
30 osteogenin or other growth factors, are bound to the
31 hollow fibers and to the fibers via the glycosaminoglycan
32 or other cross-linking agent.

1 The hollow fiber bioreactor is a component of a
2 hollow fiber cell culture system. A typical hollow fiber
3 cell culture system, such as the CELLMAX™ 100 hollow
4 fiber cell culture system (Cellco Advanced Bioreactors,
5 Inc., Kensington, MD.), which is described in Knazek *et*
6 *al.* U.S. Patent Application No. 07/238,445, supra.,
7 which disclosure is herein incorporated in its entirety
8 by reference thereto, consists of a standard glass media
9 bottle, which serves as the reservoir, stainless
10 steel/Ryton gear pump, an autoclavable hollow fiber
11 bioreactor, which consists of the fibers and shell casing
12 in which cells are cultured, and medical grade silicone
13 rubber tubing, or other connecting means, which serves as
14 a gas exchanger to maintain the appropriate pH and pO₂ of
15 the culture medium. All components are secured to a
16 stainless steel tray of sufficiently small dimensions to
17 enable four such systems to fit within a standard tissue
18 culture incubator chamber. The pump speed and automatic
19 reversal of flow direction are determined by an
20 electronic control unit which is placed outside of the
21 incubator and is connected to the pump motor via a flat
22 ribbon cable which passes through the gasket of the
23 incubator door. The pump motor is magnetically coupled
24 to the pump and is lifted from the system prior to steam
25 autoclaving.

26 Tissue culture medium, which may, for example,
27 include growth promoting substances, such as IL-2, and/or
28 recombinant vectors, is drawn from the reservoir, pumped
29 through the lumina of the hollow fibers, and then passed
30 through the gas exchange tubing in which it is re-
31 oxygenated and its pH readjusted prior to returning to
32 the reservoir for subsequent recirculation. The order

1 of sequences may be altered without substantially
2 changing teh functionality.

3 The flow rate can be increased as the number of
4 cells increases with time. Typically the initial flow
5 rate of the medium is adjusted to about 40 ml./min. The
6 direction of perfusion of the medium through the hollow
7 fiber lumina may be periodically and automatically
8 reversed, typically every ten minutes, in order to
9 provide a more uniform distribution of nutrient supply,
10 waste dilution, and cells within the space surrounding
11 the hollow fibers.

12 The entire system is sterilized prior to cell
13 inoculation and is designed for operation in a standard
14 air-CO₂ tissue culture incubator. Upon inoculation, the
15 cells settle onto the surface of the hollow fibers,
16 through which nutrients pass to feed the cells and
17 through which metabolic waste products pass and are
18 diluted into the large volume of the recirculating
19 perfusate. The selected fiber should be semi-permeable
20 to permit the passage of nutrients into the EFS and
21 should be of a material on which or in the vicinity of
22 which the cells are able to grow. The fibers are made of
23 material, such as DEAE-cellulose or polypropylene, that
24 is semi-permeable or porous and suitable for the growth
25 of mammalian cells. For example, cellulosic hollow
26 fibers 12 inches in length, whose walls nominally
27 restrict diffusion to substances having a molecular
28 weight less than 3000 Daltons are suitable for use in
29 practicing this invention.

30 In some embodiments of this invention components
31 of bone marrow or bone marrow cultures, such as stromal
32 cells or glycosaminoglycans, including heparin, to which

1 growth factors adsorb, may be bound to the fibers, either
2 reversibly or irreversibly. Endogenously produced growth
3 factors will then bind to the fibers. Alternatively,
4 exogenous growth factors, such as the interleukins,
5 colony stimulating factors, and osteogenin, can be added
6 to the EFS. The bone marrow cells are then introduced
7 into the EFS and are cultured in an environment that
8 mimics the in vivo environment. Binding may be
9 reversible, such as by adsorption, or irreversible if a
10 cross-linking agent is used to permanently affix the
11 component or the growth promoting substance to the fiber.
12 Alternatively, the growth promoting substance may also be
13 included in the perfusate and/or in the EFS.

14 A suspension of cells is inoculated into the
15 extra-fiber space (EFS) of a hollow fiber bioreactor
16 typically through one of two side ports. The lumina are
17 perfused with cell culture medium and the cells are
18 maintained in vitro for the desired period of time.

19 Upon inoculation into the EFS it is important
20 that an adequate supply of oxygen is provided to the
21 cells in order to prevent hypoxia, which predisposes stem
22 cells to commit irreversibly to the erythropoietic
23 pathway. Relatively low flow rates are used in order to
24 prevent the bone marrow cells and/or the adherent stromal
25 cells, which are loosely adherent, from washing off the
26 fibers as a result of EFS bulk flow and to prevent
27 displacement of diffusible or poorly diffusible paracrine
28 secreted products from the microenvironment of the target
29 cells, thereby preventing them from being maintained,
30 suppressed or stimulated.

31 In addition, a small circulating volume of
32 medium should be used initially so that the diffusible

1 nutrient paracrine factors are not diluted to too great
2 an extent.

3 Relatively low incubator temperatures may be
4 needed (32° C to 33° C) for maintenance of the culture.

5 The EFS should not be disturbed. Removing the
6 marrow from the EFS to examine the cells disturbs the
7 microenvironment and may trigger the commitment process.

8 In certain embodiments of this invention, the
9 methods of this invention, may be used for culturing bone
10 marrow obtained from healthy donors or from patients
11 suffering from disorders, such as cancers that have not
12 metastasized to the bone marrow, that do not involve the
13 bone marrow. Such bone marrow, herein referred to as
14 normal marrow, may be used for autologous or allogenic
15 transplants.

16 In these embodiments, a sufficient amount of
17 bone marrow, which is usually washed and separated from
18 erythrocytes using standard clinical methods, is
19 inoculated into a hollow fiber bioreactor, such as the
20 CELLMAX™ bioreactor, and cultured until it is needed for
21 transplantation, such time is generally at least two to
22 four weeks, but may be substantially longer. During this
23 time the proportion of pluripotent stem cells remains
24 substantially constant or increases compared the
25 proportion of such cells in the inoculum. In contrast
26 to bone marrow cells cultured by the methods of the prior
27 art, the proportion of stem cells initially present in
28 the bone marrow usually does not substantially decrease.
29 The cells are then harvested from the bioreactor and
30 transfused into the recipient.

31 In other embodiments of this invention the
32 methods of this invention may be used for clearing

1 leukemic and/or other cancerous cells from the marrow in
2 vitro. In instances in which malignant cells are
3 present in the marrow it is may be advantageous to
4 destroy such cells by chemotherapy and/or radiation
5 therapy. Prior to treatment, a sample of marrow, of
6 about, although not limited to, 400 to 800 ml., is
7 aspirated from the patient. If desired, erythrocytes can
8 be removed using standard well-known methods. After
9 aspiration, the marrow can be treated with agents that
10 destroy diseased cells, after which treatment the
11 remaining cells are inoculated into a hollow fiber
12 bioreactor. Alternatively the marrow can be introduced
13 into a hollow fiber culture bioreactor without treatment.

14 In one embodiment of this invention, marrow is
15 obtained from a patient suffering from a leukemia, such
16 as CML, which exhibits detectable phenotypic or genotypic
17 markers, such as chromosomal translocations that may be
18 visible upon microscopic inspection or detected by
19 methods such as hybridization with probes that span the
20 breakpoint (see, e.g., U.S. Patent No. 4,701,409 to Croce
21 et al.).

22 After aspiration the marrow is inoculated into
23 a hollow fiber bioreactor, such as the CELLMAX™ 100
24 bioreactor, and is cultured. After a sufficient period
25 of time in culture in the bioreactor, which is generally,
26 but not limited to, about four weeks, the leukemic cells
27 are cleared from the culture. The cultured cells are
28 then harvested from the bioreactor and reinfused into the
29 patient.

30 In other embodiments of this invention, the bone
31 marrow cells may be co-cultured with or pretreated with
32 TIL and/or LAK cells, which are prepared from the same

1 patient or which are known to specifically react with the
2 leukemic cells of the patient. The TIL and or LAK cells
3 may be induced upon inoculation of the bone marrow into
4 the bioreactor by the addition of an effective
5 concentration of a growth promoting substance, such as
6 IL-2, to the EFS and/or perfusate. Alternatively, the
7 bone marrow cells may be treated with or cultured in the
8 presence of chemotherapeutic agents that destroy the
9 cancerous cells or that enhance the ability of immune
10 cell in the marrow to destroy cancer cells.

11 In the methods involving co-culturing with TIL
12 and/or LAK cells or culturing in the presence of
13 chemotherapeutic agents, the cells or agents may
14 be introduced into the perfusing medium or into the EFS.
15 If introduced into the EFS, the cells or agents may be
16 bound, reversibly or irreversibly, to the surfaces of the
17 fibers.

18 In other embodiments of this invention, the
19 methods of this invention are used to clear solid tumor
20 cells from bone marrow. During progression of diseases,
21 such as breast, prostate, lung and other organ cancers,
22 tumor cells frequently metastasize to the bone marrow.
23 When this occurs, the cancer is almost uniformly
24 terminal because any therapies, such as chemotherapy or
25 radiation therapy, that destroys the metastatic foci
26 also destroys the bone marrow. Aspiration of bone
27 marrow prior to this therapy protects such cells from
28 toxic effects.

29 In this invention, the marrow from a patient
30 suffering from metastatic cancer is introduced into a
31 bioreactor and is cultured in the presence of a mitogen,
32 such as an interleukin, that specifically expands tumor-

1 specific T-lymphocytes (see, co-pending U.S. Patent
2 Application No. 07/407,456 to Knazek et al., supra.),
3 which cells specifically inactivate the tumor cells and
4 thereby clearing the co-cultured marrow of tumor cells.
5 After a sufficient time for such clearing to occur has
6 elapsed, the tumor-free bone marrow cells are harvested
7 and reinfused into the patient, who has been treated with
8 chemotherapy and/or radiation to destroy tumor cells.

9 In one embodiment of this invention, marrow
10 containing metastatic tumor cells is withdrawn from the
11 patient and inoculated into the EFS of a CELLMAX™
12 bioreactor. IL-2 is also inoculated into the EFS and/or
13 is included in the perfusing medium. Alternatively,
14 TIL and/or LAK cells are inoculated into the EFS.
15 After about 2 to 3 weeks the bone marrow cells are
16 harvested from the bioreactor and reinfused into the
17 patient.

18 In other embodiments of this invention, bone
19 marrow cells are transfected with a recombinant vector,
20 which includes DNA encoding a gene product and which is
21 capable of expressing this DNA in mammalian cells, to
22 produce recombinant bone marrow stem cells for gene
23 therapy. Upon infusion of the stem cells a gene product
24 is permanently provided to the recipient.

25 The preparation and selection of the vector and
26 DNA encoding at least one gene product is within the
27 level of skill in the art. In general, the vector will
28 be a viral vector that can be replicated and packaged by
29 selected target cells but not by bone marrow cells. It
30 may be a vector that is integrated into a host cell
31 genome, such as a retrovirus-derived vector, or one, such
32 as vector derived from Epstein Barr virus, that remains

1 episomal. The gene product may be a therapeutic product,
2 such as an anti-cancer or anti-viral agent; it may be a
3 product, such as adenosine deaminase or immunoglobulin,
4 that the recipient fails to produce or produces in a
5 mutated defective form because of a genetic defect; it
6 may be a marker, such as DNA that encodes neomycin or
7 methotrexate resistance, whereby the reinfused bone
8 marrow cells may be selected or detected, or any other
9 gene product.

10 In this embodiment, the selected recombinant
11 vector is introduced by transfection or any other method
12 known to those of skill in the art into a convenient
13 target host cell, which is capable of replicating and
14 packaging the vector at high titer. The target host
15 cell is then cultured in a bioreactor, such as the
16 CELLMAX™ bioreactor for a time and under conditions
17 whereby the vector is released into the EFS, which then
18 contains high titers of the recombinant vector. The EFS
19 is then harvested batchwise, periodically, or
20 continuously by connecting it to the EFS of a second
21 bioreactor.

22 Bone marrow cells are removed from a donor, who
23 is preferably the intended recipient of the cultured
24 modified bone marrow cells, and introduced into the
25 second bioreactor, such as the CELLMAX™ bioreactor. The
26 harvested EFS that contains the recombinant viral vector
27 is introduced into the EFS of the bioreactor that
28 contains the bone marrow cells or is mixed with the bone
29 marrow cells prior to introduction into the bioreactor.
30 This step can be repeated a plurality of times in order
31 to insure that a high percentage of the pluripotent stem
32 cells take up or are transfected with the recombinant

1 DNA. Alternatively, the EFS that contains the vector may
2 be included in the perfusing medium, if hollow fibers
3 having a sufficiently large pore size to permit diffusion
4 of the vector into the EFS.

5 The bone marrow cells are cultured for at least
6 about two to four weeks, whereby stem cells that contain
7 the recombinant vector in a stable manner are produced
8 and/or maintained. The cultured bone marrow cells are
9 then harvested and infused into a recipient.

10 In some embodiments, the recipient is treated
11 with chemotherapy and/or radiation to destroy his or her
12 bone marrow cells, prior to infusion of the recombinant
13 stem cells. In other embodiments, in which the vector
14 includes a selective marker, such as drug resistance, the
15 recipient may be treated with the drug prior to infusion
16 and/or after infusion of the selective-marker-modified
17 bone marrow cells.

18 As a first step when practicing any of the
19 embodiments of the invention disclosed herein bone marrow
20 cells must be removed from an individual. Such individual
21 is generally the patient who is to be treated using an
22 adoptive immunotherapeutic method or a matched donor.

23 The cells obtained from the patient or donor
24 are suspended in any cell culture medium that is suitable
25 for sustaining the growth of such mammalian cells. Such
26 media are readily available and the choice of an
27 appropriate medium is well within the level of skill in
28 the art.

29 The cells may be treated to remove the
30 erythrocytes and are then suspended in the tissue culture
31 medium at a suitable concentration, which is about, but
32 is not limited to, 10^5 to 10^7 cells per ml.

1 A sufficient volume of cells to fill the EFS of
2 a bioreactor cartridge is inoculated into the pre-
3 sterilized cartridge and placed in an incubator at an
4 appropriate temperature, generally about 32° C to about
5 37° C and maintained under these conditions for up to
6 several months.

7 The conditions, including temperature and media,
8 are selected whereby the relative proportion of
9 pluripotent stem cells remains constant or increases.

10 After about 3 to 7 days in the EFS erythroid
11 colonies appear, which is evidenced by the appearance of
12 reddening of the previously white streaks of cells. This
13 suggests that a percentage of the cells have committed to
14 erythroid differentiation shortly before or after
15 inoculation.

16 After inoculation, the culture medium is
17 continuously perfused through the hollow fiber bioreactor
18 by means of externally applied pressure, such as a pump.
19 A glass reservoir, the hollow fiber bioreactor, and
20 pumping means are connected by tubing, typically silicone
21 rubber, an hollow fiber oxygenator or other means of
22 oxygenating media known to those of skill in the art,
23 which simultaneously serves as a membrane gas exchanger
24 to replenish oxygen and, if the medium is buffered with
25 bicarbonate, to maintain the pH via CO₂ transport into the
26 perfusion medium. Medium that is buffered with systems
27 other than bicarbonate do not necessarily require CO₂ in
28 the incubator.

29 As the cells are cultured, the perfusate can be
30 replaced. Typically, it is replaced about once a week.
31 Care must be taken not to disturb the cells in the EFS.
32 Disturbances to these cell may cause them to become

1 committed multipotent and end-stage cells.

2 The perfusing medium can be replenished by
3 replacing the reservoir bottle with one containing fresh
4 medium. After growth of the cells has been established,
5 the cells can be harvested by gently shaking the
6 bioreactor and pouring the suspended cells into a side
7 port bottle. In addition, the EFS cell supernatant,
8 which is rich non-or poorly-diffusible cellular products,
9 including useful biologically active agents, cytokines
10 and lymphokines produced by the cultured cells, can be
11 recovered for further processing in order to purify or
12 partially purify said biologically active agents. The
13 cells can be spun down using a centrifuge or by any other
14 means known to those of skill in the art to yield a cell
15 pellet and the EFS cell supernatant, which is enriched in
16 biologically active molecules, such as growth-promoting
17 substances.

18 The harvested cells can be assayed for the
19 presence of normal pluripotent stem cells using standard
20 semi-solid colony assays. The harvested cells can also
21 be transplanted into a recipient.

22 After harvesting and pelleting the cells, the
23 EFS cell supernatant may be dialyzed against fresh tissue
24 culture medium in order to produce EFS conditioned
25 medium. The conditioning factors may also be isolated
26 or partially purified using standard well-known protein
27 purification methods.

28 In typical procedures using the methods of this
29 invention, bone marrow cells are harvested from a patient
30 suffering from CML or from a healthy bone marrow donor.
31 All operations in which the cells are manipulated are
32 performed using sterile techniques in a laminar flow

1 hood.

The erythrocytes are removed using standard clinical methods and the remaining cells are suspended in suitable tissue culture medium, such as AIM-V, at a density of about 10^7 and 10^8 cells per ml. About 50 ml. of the suspension is inoculated into a single bioreactor cartridge.

Prior to use the hollow fiber culture system is steam autoclaved, continuously perfused with 1.3 liters of recirculating deionized water, drained, flushed, and perfused with the selected tissue culture medium in both the EFS and perfusate pathways.

13 The inoculated bioreactor is transferred to a
14 standard incubator where it is perfused with medium. If
15 the cells are to be modified for use in genetic therapy,
16 a high concentration of the selected recombinant vectors,
17 containing the heterologous DNA, is added to the EFS.
18 The vector may be added to the EFS continuously or a
19 plurality of times during the incubation period or it may
20 be added to the EFS via the perfusing medium if hollow
21 fibers that have a sufficiently high pore size to permit
22 diffusion of the vector are selected.

If TIL cells are desired, such as in embodiments in which the bone marrow is obtained from a patient suffering from metastatic cancer, they are added to the EFS or induced to proliferated or are activated by the addition of an appropriate growth-promoting substance, such as IL-2 or anti-CD3 monoclonal antibody, to the EFS and/or to the perfusing medium. Additionally, any desired chemotherapeutic agents that destroy malignant cells may be added to the EFS and/or perfusing medium.

1 Incubation continues for at least about one to
2 thirty days. During the incubation period the reservoir
3 containing the perfusing medium is replaced in order to
4 maintain a sufficiently high concentration of glucose and
5 other diffusible nutrients in the EFS and for waste
6 removal.

7 When the incubation period is complete, the
8 cells are harvested by shaking the hollow fiber
9 bioreactor and draining the EFS. The cells are pelleted
10 and the EFS cell supernatant collected for further
11 processing.

12 If the bone marrow cells were obtained from a
13 healthy donor or from a patient suffering from a
14 leukemia, the harvested cells will most likely be non-
15 leukemic and should contain a greater proportion of
16 pluripotent stem cells than did the inoculum.

17 The following examples are included for
18 illustrative purposes only and are not intended to limit
19 the scope of the invention.

20 EXAMPLE 1

21 The CELLMAX™ 100 bioreactor system was used for
22 the bone marrow cultures with either a B3 or B4 hollow
23 fiber bioreactor. Prior to use the silicone rubber
24 tubing flow path was connected to the pump and reservoir
25 and steam autoclaved with side port tubing and bottles at
26 121° C for 20 minutes.

27 Each hollow fiber bioreactor cartridge was
28 sterilely removed from its package and sterilely inserted
29 into the sterilized silicone rubber tubing pathway. The
30 side port bottles were attached to the side ports. Each
31 bioreactor was also, on occasion, steam autoclaved
32 simultaneously with the perfusion path after having been

1 inserted into the perfusion flow path. During this
2 procedure the cart-ridge was kept full of distilled
3 water, because allowing the fiber alters their ability to
4 support cell growth.

After sterilization, the distilled water in the EFS of the bioreactor was drained into sideport bottles, discarded, and replaced with complete culture medium. The reservoir was also filled with complete medium and the entire system was perfused overnight in a humidified 5% CO₂ incubator at 37° C. Media that were used include: AIM-V, Iscove's.

12 Bone marrow cells, which were obtained from
13 patients or paid volunteers and were cleared of red cells
14 using standard clinical methods. The cells were
15 inoculated into the EFS via the side port bottles to fill
16 the EFS.

17 The entire CELLMAX™ bioreactor unit was then put
18 into the incubator, but not perfused for 15 hours in
19 order to facilitate attachment of cells to the fibers.
20 Subsequently, perfusion was commenced at a rate of about
21 40 ml per minute. The perfusion medium was replaced at
22 intervals during the period approximately once a week.
23 Care was taken to avoid disturbing the cells.

After culturing the cells for several weeks, the bone marrow cells are harvested for subsequent infusion into the recipient.

EXAMPLE 2

28 Bone marrow cells were obtained from a patient
29 having from CML. As determined by standard cytological
30 techniques, virtually 100% of the cells exhibited the
31 characteristic Philadelphia chromosome. The cells were
32 inoculated into the bioreactor and cultured in AIM-V

1 medium as described in Example 1 except that four days
2 after inoculation, the incubator temperature was
3 decreased from 37° C to 33° C.

4 Four weeks after inoculation, an aliquot of the
5 EFS was removed and the cells therein were assayed for
6 the presence of the Philadelphia chromosome. Eleven
7 mitotic figures were examined and none exhibited the
8 Philadelphia chromosome.

9 EXAMPLE 3

10 Approximately 5×10^7 cells from bone marrow that
11 had been harvested from a normal volunteer were
12 inoculated into a B3 cartridge through which with
13 Iscove's medium was perfusing.

14 A colony-forming assay was also performed on an
15 aliquot of bone marrow cells at the time of harvest from
16 the patient. This revealed the following after 14 days
17 of culture in agar:

18 CFU-GM: 0.2 per 10^5 cells plated
19 BFU-F: 0.1 per 10^5 cells plated.

20 On day 13, a 10 ml aliquot of cell suspension
21 was removed from the EFS. Trypan blue dye exclusion
22 revealed that the sampled cells were 100% viable. The
23 next day a 5 aliquot was removed from the EFS and assayed
24 by means of the colony-forming assay. After fourteen
25 days of culture in agar there were:

26 CFU-GM: 55 per 10^5 cells plated
27 BFU-E: 21 per 10^5 cells plated.

28 These results indicated that the pluripotent
29 cells present in the harvested cells proliferated during
30 the fourteen days of culture within the bioreactor.

1 We claim:

2 1. A method for culturing bone marrow cells in
3 vitro, comprising:

4 (a) inoculating the extra fiber space of a
5 hollow fiber bioreactor that is a component of a hollow
6 fiber culture system with a suspension of bone marrow;
7 and

8 (b) incubating said suspension in said
9 bioreactor, whereby at least a portion of the pluripotent
10 stem cells in said suspension proliferate or are
11 maintained.

12 2. The method of claim 1, wherein the
13 proportion of pluripotent stem cells in said suspension
14 remains substantially constant or increases compared to
15 the proportion of said cells in the inoculum.

16 3. The method of claim 1, wherein said bone
17 marrow suspension also contains neoplastic cells

18 4. The method of claim 3, where in said
19 neoplastic cells are selected from the group consisting
20 of leukemic cells or cancerous cells from a solid tumor.

21 5. The method of claim 3, wherein an effective
22 amount of at least one growth promoting substance that
23 specifically expands a therapeutically useful
24 subpopulation of lymphoid cells is added to the extra
25 fiber space (EFS) of said bioreactor and wherein said
26 effective amount is an amount sufficient to effect said
27 specific expansion and said subpopulation is effective in
28 inactivating at least a portion of said neoplastic cells.

29 6. The method of claim 1, wherein an effective
30 amount of at least one growth promoting substance is
31 added to the extra fiber space (EFS) or perfusate of said
32 bioreactor, wherein said effective amount is an amount

1 sufficient to effect expansion of the number of
2 pluripotent or multipotent cells in said bone marrow
3 suspension.

4 7. The method of claim 6, wherein the growth
5 promoting substance is at least one substance
6 selected from the group consisting of mitogens and
7 cytokines.

8 8. The method of claim 7, wherein said growth
9 promoting substances is a least one substance selected
10 from the group of cytokines and mitogens consisting of
11 granulocyte-macrophage colony stimulating factor,
12 granulocyte colony stimulating factor, interleukin-1,
13 interleukin-2, interleukin-3, interleukin 4, interleukin
14 6 and osteogenin.

15 9. The method of claim 3, wherein an effective
16 amount of at least one growth promoting substance
17 that specifically expands a therapeutically useful
18 subpopulation of lymphoid cells is included in
19 the tissue culture medium that perfuses bioreactor and
20 wherein said effective amount is an amount sufficient to
21 effect said specific expansion and said subpopulation is
22 effective in inactivating at least a portion of said
23 neoplastic cells.

24 10. The method of claim 5, wherein the growth
25 promoting substance is at least one substance
26 selected from the group consisting of mitogens and
27 cytokines.

28 11. The method of claim 9, wherein the growth
29 promoting substance is at least one substance
30 selected from the group consisting of mitogens and
31 cytokines.

1 12. The method of claim 10, wherein said growth
2 promoting substances is a least one substance selected
3 from the group of cytokines and mitogens consisting of
4 granulocyte macrophage colony stimulating factor,
5 granulocyte colony stimulating factor, interleukin-1,
6 interleukin-2, interleukin-3, interleukin 4, interleukin
7 6 and osteogenin.

8 13. The method of claim 11, wherein said growth
9 promoting substance is a least one substance selected
10 from the group of cytokines and mitogens consisting of
11 granulocyte-macrophage colony stimulating factor,
12 granulocyte colony stimulating factor, interleukin-1,
13 interleukin-2, interleukin-3, interleukin 4, interleukin
14 6 and osteogenin.

15 14. The method of claim 1, further comprising
16 harvesting the contents of the extra fiber space of the
17 bioreactor, wherein said contents include said
18 pluripotent stem cells and the extra fiber space cell
19 supernatant.

20 15. The method of claim 3, further comprising
21 harvesting the contents of the extra fiber space
22 of the bioreactor, wherein said contents includes
23 said pluripotent stem cells and the extra fiber space
24 cell supernatant.

25 16. The method of claim 5, further comprising
26 harvesting the contents of the extra fiber space
27 of the bioreactor, wherein said contents include
28 said pluripotent stem cells, in vitro expanded
29 lymphoid cells and the extra fiber space cell
30 supernatant.

31 17. The method of claim 15, further comprising
32 pelleting and removing the cells from said contents

1 of the extra fiber space to produce an extra fiber
2 space cell supernatant.

3 18. The method of claim 16, further comprising
4 pelleting and removing the cells from said contents of
5 the extra fiber space to produce an extra fiber space
6 cell supernatant.

7 19. The method of claim 1, further comprising
8 adding at least one component of bone marrow or bone to
9 the extra fiber space (EFS) of said bioreactor prior to
10 said incubation
11 step.

12 20. The method of claim 19, wherein said
13 component is derived from the bone marrow extracellular
14 matrix.

15 21. The method of claim 20, wherein said
16 component is selected from the group consisting
17 of glycosaminoglycans.

18 22. The method of claim 19, further comprising
19 adsorbing at least one growth promoting substance to
20 said component.

21 23. The method of claim 19, wherein an effective
22 amount of at least one growth promoting substance is
23 added to the extra fiber space (EFS) or perfusate of
24 said bioreactor, wherein said effective amount is an
25 amount sufficient to effect said specific expansion the
26 pluripotent or multipotent cells in said bone marrow
27 suspension.

28 24. The method of claim 22, wherein the growth
29 promoting substance is at least one substance
30 selected from the group consisting of mitogens and
31 cytokines.

1 25. The method of claim 24, wherein said growth
2 promoting substances is a least one substance
3 selected from the group of cytokines and mitogens
4 consisting of granulocyte-macrophage colony stimulating
5 factor, granulocyte colony stimulating factor,
6 interleukin-1, interleukin-2, interleukin-3, interleukin
7 4, interleukin 6 and osteogenin.

8 26. A method of adoptive immunotherapy for the
9 treatment of cancer, comprising:

10 (a) obtaining from a donor bone marrow
11 that contains at least an effective number of pluripotent
12 stem cells;

13 (b) inoculating the extra fiber space of a
14 hollow fiber bioreactor that is a component of a hollow
15 fiber culture system with a suspension of said bone
16 marrow cells; and

17 (c) incubating said cells in said bioreactor
18 under conditions in which said cells remain viable,
19 whereby at least some of the pluripotent stem cells of
20 said bone marrow cells proliferate or retain the ability
21 to differentiate, wherein said effective number is
22 capable of reconstituting the bone marrow of a recipient
23 of said cells after said cells have been cultured.

24 27. The method of claim 26, further comprising
25 harvesting said cells following step (c) and
26 infusing said cells into a recipient, whose bone marrow
27 has been destroyed, substantially depleted or is
28 defective.

29 28. The method of claim 27, wherein said
30 recipient is suffering from a neoplastic disease.

31 29. The method of claim 28, wherein said
32 recipient is the same individual as said donor and

1 wherein said bone marrow cells were obtained prior to
2 destruction of, depletion of, or damage to the bone
3 marrow.

4 30. The method of claim 29, wherein an
5 effective amount of at least one growth promoting
6 substance that specifically expands a therapeutically
7 useful subpopulation of lymphoid cells is added to the
8 extra fiber space (EFS) of said bioreactor, wherein said
9 effective amount is an amount sufficient to effect said
10 specific expansion and said subpopulation is effective in
11 inactivating said neoplastic cells.

12 31. The method of claim 29, wherein an
13 effective amount of at least one growth promoting
14 substance that specifically expands a therapeutically
15 useful subpopulation of lymphoid cells is included in the
16 tissue culture medium that perfuses said bioreactor,
17 wherein said effective amount is an amount sufficient to
18 effect said specific expansion and said subpopulation is
19 effective in inactivating said leukemic or cancerous
20 cells.

21 32. A method of introducing heterologous DNA
22 into pluripotent or multipotent stem cells, comprising:

23 (a) adding a suspension that contains a high
24 concentration of a recombinant vector that contains
25 heterologous DNA encoding at least one gene product to a
26 bioreactor that been inoculated with a suspension of bone
27 marrow cells; and

28 (b) incubating said bioreactor under conditions,
29 whereby said pluripotent or multipotent stem cells are
30 transfected by said vectors and said DNA is stably
31 incorporated into said stem cells,

1 wherein said concentration is sufficiently high to
2 transfect at least a portion of said cells and said DNA
3 encodes a protein that is expressed when said cells are
4 used in adoptive immunotherapy.

5 33. The method of claim 32, wherein said
6 heterologous DNA encodes at least one protein selected
7 from the group consisting of traceable marker proteins,
8 therapeutically effective proteins, and proteins
9 responsible for drug resistance or sensitivity.

10 34. A method of genetic therapy, comprising
11 harvesting the bone marrow cells of claim 32 and infusing
12 a recipient with said cells.

13 35. A method for clearing neoplastic cells from
14 bone marrow, comprising culturing bone marrow cells that
15 contain said neoplastic cells in a hollow fiber
16 bioreactor.

17 36. A method for preparing bone marrow cell
18 conditioned medium for use in stimulating the growth of
19 cells and as a source of biologically active growth
20 promoting substances, comprising removing the contents of
21 the extra-fiber space of a bioreactor in which bone
22 marrow cells have been cultured, pelleting and removing
23 the cells from said contents of the extra fiber space to
24 produce an extra fiber space cell supernatant.

25 37. The cultured bone marrow cells that are
26 produced by the method of claim 35.

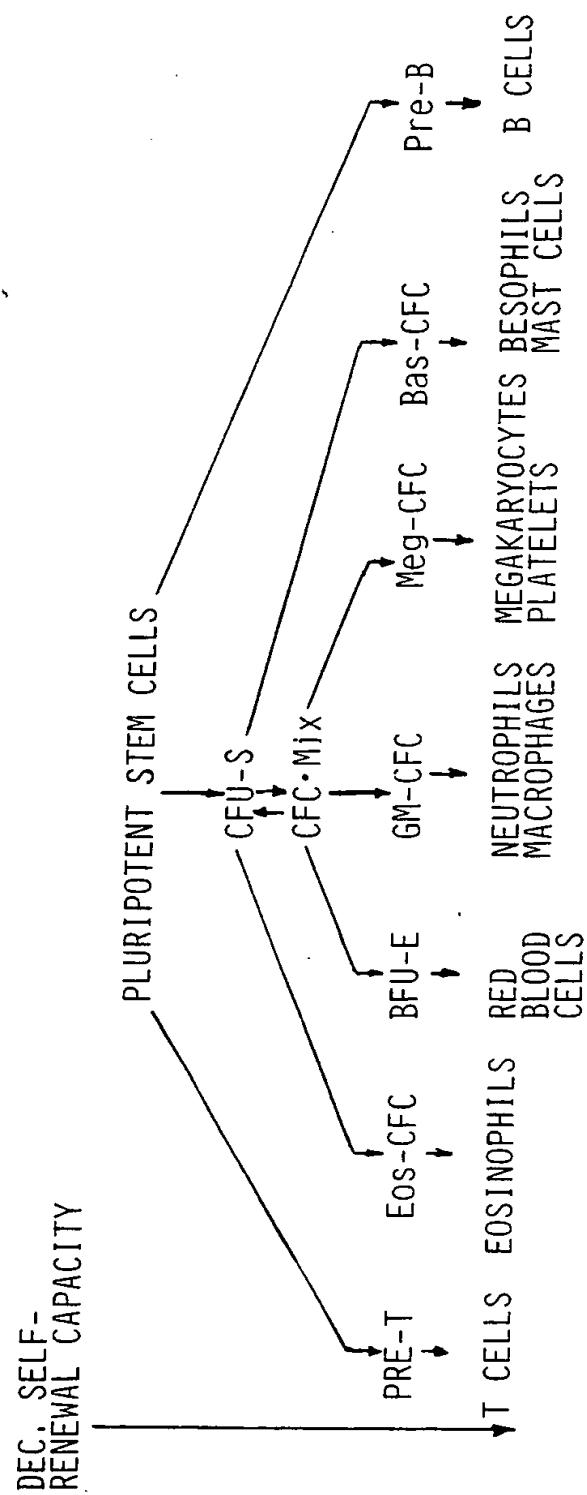
27 38. The bone marrow cell conditioned medium
28 that is produced by the method of claim 36.

29 39. The cultured bone marrow cells that are
30 produced by the method of claim 1.

31 40. The cultured bone marrow cells that are
32 produced by the method of claim 2.

- 1 41. The cultured bone marrow cells that are
- 2 produced by the method of claim 3.
- 3 42. The cultured bone marrow cells that are
- 4 produced by the method of claim 4.
- 5 43. The cultured bone marrow cells that are
- 6 produced by the method of claim 5.
- 7 44. The cultured bone marrow cells that are
- 8 produced by the method of claim 6.
- 9 45. Cultured bone marrow cells, comprising the
- 10 pluripotent and multipotent stem cells that are produced
- 11 by method of claim 32.

FIG. 1

**SUBSTITUTE SHEET**

2 / 2

FIG. 2



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US91/03555

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/06, 5/10, 5/00, 15/63, 15/65

U.S.Cl: 435/240.242, 240.2, 240.3; 424/577; 935/55,62,70

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.Cl.	435/240.242, 240.2, 240.3; 424/577; 935/55,62,70

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A, 4,220,725 (Knazek, et al) 02 September 1980. See column 4, example 1, first paragraph.	1, 19
Y	WO, A, 87/06610 (Melinck et al.) 05 November 1987. See entire document, especially abstract and claims 1-3 and 5 -7.	2-18, 20-45
Y	The Lancet, Issued 08 February 1986, Chang, et al. "Reconstitution of Haemopoietic System with Autologous Marrow Taken During Relapse of Acute Myeloblastic Leukaemia and Grown In Long-Term Culture." Pages 294-295. See 294, "Summary", and paragraph bridging pages 294 and 295	1-45

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

24 July 1991

Date of Mailing of this International Search Report

28 AUG 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

George Elliott

III DOCUMENTS CITED AS BEING RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Reference to Claims No.
Y	Blood, volume 75, number 5, issued 01 March 1990, Williams et al. Clonal Growth Of Murine Pre-B Colony-Forming Cells and Their Targeted Infection By A Retroviral Vector: Dependence on Interleukin-7. pages 1132-1138. See abstract; page 1132, left-hand column, second paragraph and page 1133, right-hand column, second and third full paragraphs.	5-8, 10-13, 22-25, 30, 31
Y	Nature, volume 236, issued 26 March 1987, Gordon, et al. *Compartmentalization Of A Hematopoietic Growth Factor (GM-CSF) By Glycosaminoglycans In The Bone Marrow Microenvironment. Pages 403-405. See title and abstract.	19-22, 24, 25
Y	Nature, volume 331, issued 07 January 1988, Dzierzak, et al. *Lineage-Specific Expression Of A Human B-Globin Gene In Murine Bone Marrow Transplant Recipients Reconstituted With Retrovirus-Transduced Stem Cells. Pages 35-41. See abstract and page 35, right-hand column, lines 21-32.	32-34, 45
Y	Nature, volume 318, issued 14 November 1985, Keller, et al. *Expression Of A Foreign Gene In Myeloid And Lymphoid Cells Derived From Multipotent Hematopoietic Precursors. pages 149-154. See entire article.	32-34, 45
Y	New England Journal Of Medicine, volume 308, issued 23 June 1983, Coulombel, et al. *Long-Term Marrow Culture Reveals Chromosomally Normal Hematopoietic Progenitor Cells In Patients With Philadelphia Chromosome-Positive Chronic Myelogenous Leukemia. Pages 1493-1498. See abstract, figure 3.	35, 37

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

British Medical Journal, volume 295, issued 07 November 1987, Dexter, "Stem Cells In Normal Growth and Disease." Pages 1192-1194. See page 1193, last full paragraph; page 1194, second full paragraph.

5-8, 10-13,
22-25, 30, 31

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter^{1,2} not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,2}, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See Attached

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. **Telephone Practice**
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. All of searchable claims could be searched without effort (without any additional fee). The International Searching Authority did not charge payment of any additional fee.

Remarks on Protest

- The required search fees were uncompensated by the protest fee.
- No protest was filed against the required additional search fees.

- I. Claims 1-**1** and 39-44 to a first method of culturing bone marrow cells, the cells so cultured and method of using the cells.
- II. Claims 32-33 to a method of introducing DNA into bone marrow cells
- III. Claim 34 to a method of using transfected bone marrow cells.
- IV. Claim 35 to a method of purging bone marrow cells of neoplastic cells.
- V. Claim 36 to a method of preparing bone marrow cell conditioned medium.
- VI. Claim 37 to bone marrow cells depleted of neoplastic cells.
- VII. Claim 38 to bone marrow cell conditioned medium.
- VIII. Claim 45 to bone marrow cells transfected with exogenous DNA.